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(54) Title: PROCESS FOR QUANTIFICATION OF NUCLEIC ACIDS

(57) Abstract

A process for the quantification of a target nucleic acid in a sample comprises the steps of: (i) adding to the sample containing said target nucleic acid a known amount of a competitor nucleic acid; (ii) amplifying both the competitor and target nucleic acids using reagents capable of amplifying the competitor and target nucleic acids in parallel; (iii) immobilizing the amplified nucleic acids onto a biosensor sensing surface; and (iv) subjecting the respective immobilized nucleic acids to a biospecific interaction or interactions, and from the changes in a property of the sensing surface caused by the interactions of the respective nucleic acids determining the relative amounts of the target and competitor nucleic acids to thereby determine the amount of said target nucleic acid in said sample.

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PROCESS FOR QUANTIFICATION OF NUCLEIC ACIDS

FIELD OF THE INVENTION

The present invention relates to nucleic acid analysis, and more particularly to a novel process for the quantification of nucleic acid fragments.

BACKGROUND OF THE INVENTION

The polymerase chain reaction (PCR) is an in vitro method of amplifying DNA sequences which is described in i.a. Mullis and Faloona, Methods Enzymol., 155: 335 (1987) and in U.S. Patents 4,683,195 and 4,683,202. In PCR, both strands of a target DNA sequence are replicated by enzymatic DNA synthesis, initiated from two oligonucleotide primers. The temperature of the reaction is varied cyclically to allow denaturation of the DNA template, followed by hybridization of the primers to the target sequence, and DNA synthesis. In each cycle the number of copies of the target segment is approximately doubled resulting in exponential amplification. The nature of PCR, as well as of related nucleic acid amplification techniques, results in comparable amounts of product, starting either with few or many template copies. This limits the use of these techniques as quantitative tools. For many diseases, a quantitative measurement is needed to make proper diagnosis and it would be an advantage to be able to measure the amount of pathogen during treatment to make a relevant prognosis. The use of competitive methods where titered amounts of an engineered and distinguishable competitor DNA are co-amplified with the wild type template, enables a determination of the initial number of target templates. The bottleneck of this strategy, however, is the problems associated with specific detection of the respective PCR products for the determination of their relative ratios. Different methods for such analysis have been described, involving quantification of differently sized DNA fragments by electrophoresis (Gilliland et al., Proc. Natl Acad. Sci. USA, 87: 2725-2729, (1990)), restriction endonuclease digestion followed by

electrophoresis (Becker-André et al., Nucleic Acids Rès., 17: 9437-9446, (1989)) or colorimetric detection using DNA binding proteins fused to reporter enzymes (Lundeberg et al., Biotechniques, 10: 68-75, (1991)). All these methods are associated with difficulties in the quantification step, either in the scanning of electrophoresis gels or the spectroscopic analysis of colour after a specified period of time. In addition, while standard techniques for the enzymatic manipulation of nucleic acids exist, such as restriction with endonucleases, splicing using ligase, and extension by polymerases (Sambrook et al., (1989) Molecular cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, N.Y.), and are routinely performed in solution in a test tube, there are no possibilities of monitoring the actual progress of the different activities.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide a process for quantitative determination of nucleic acid species, which process does not suffer from the above disadvantages, is convenient to perform and gives reliable results.

According to the present invention, the above and other objects and advantages are obtained by a process for the quantification of a target nucleic acid in a sample, which process comprises the steps of:

(i) adding to the sample containing said target nucleic acid a known amount of a competitor nucleic acid;

(ii) amplifying both the competitor and target nucleic acids using reagents capable of amplifying the competitor and target nucleic acids in parallel;

(iii) immobilizing the amplified nucleic acids onto a biosensor sensing surface; and

(iv) subjecting the respective immobilized nucleic acids to a biospecific interaction or interactions, and from the changes in a property of the sensing surface caused by the interactions of the respective nucleic acids determining the relative amounts of the target and

competitor nucleic acids to thereby determine the amount of said target nucleic acid in said sample.

The term "nucleic acid" includes DNA and RNA.

The term "competitor nucleic acid" means a nucleic acid which is similar to the target nucleic acid and can be amplified using the same reagents, but which in some part differs from the target nucleic acid sequence to permit target and competitor nucleic acid fragments to be distinguished form each other by biospecific interactions.

Preferably, the amplification step (ii) comprises using oligonucleotide primers capable of hybridising to sequences shared by the two nucleic acid species.

DETAILED DESCRIPTION OF THE INVENTION

A typical application of the present invention comprises amplification of the sample nucleic acids in parallel with an amplification of a titered amount or a series of titered amounts of related nucleic acids, or competitor nucleic acids, recruiting the same reagents for amplification. The amplification procedure can be any of several existing *in vitro* or *in situ* nucleic acid amplification procedures (Abramson et al., (1993) Curr. Opin. Biotechnol., 4: 41-47), typically the polymerase chain reaction (PCR)(Saiki et al., (1988) Science, 239: 487-491), ligase chain reaction (LCR) (Barany, F., et al., (1991) Proc. Natl. Acad. Sci. USA, 88: 189-193; Barany, F., et al., (1991) PCR Meth. Appl., 1: 5-16), Q_b replicase amplification (Fox et al., (1989) J. Clin. Lab. Anal., 3: 378-87) or NASBA (Kievits et al., (1991) J. Virol. Methods, 35: 273-286). The resulting amplification product contains a mixture of amplicons originating from the target nucleic acids and the added competitor nucleic acid, and the numeral ratio of these resulting nucleic acid species reflects the initial stoichiometric relationship between the two nucleic acid species. Since the amount of competitor nucleic acids added is known, the initial number of target nucleic acids molecules can be determined if the ratio between the number of resulting amplicons can be determined. This determination can be performed employing

hybridisations, fragment extensions or shortenings monitored by biospecific interaction analysis, preferably in real-time.

A basic feature of the present invention is that the amplified nucleic acid fragments are immobilized onto a suitable solid support utilizing a suitable coupling chemistry or biology. Examples of such coupling techniques are the use of the high affinity interaction between biotin and streptavidin or avidin (Wilchek et al., Anal. Biochem., 171: 1-32, (1988)), thiol coupling (Ljungqvist et al., Eur. J. Biochem., 186: 557-561, (1989)) or interactions between an antibody and a hapten (Digoxigenin, Boehringer Mannheim, Germany).

Another basic feature of the present invention is the use of biosensor-based detection means to monitor, preferably in real-time, the further enzymatic or other manipulation of the immobilized nucleic acid fragments. Such detection means may advantageously be taken from recent development in instrumentation for biospecific interaction analysis (BIA), i.e. biosensor technology (Jönsson et al, Biotechniques, vol. 11, 5: 620-627, (1991)).

Thus, in accordance with the present invention the nucleic acid fragments are immobilized onto a solid phase and the quantification of the same is monitored by means of biosensor technology. A biosensor may be defined as being a unique combination of a receptor for molecular recognition and a transducer for transmitting the interaction information to processable signals. In one type of biosensor, the receptor is supported on a sensing surface and molecular interaction with the receptor is detected as a change of a property of the sensing surface. Exemplary of such biosensor technologies are those based on mass detecting methods, such as piezoelectric, optical, thermo-optical and surface acoustic wave (SAW) methods, and electrochemical methods, such as potentiometric, conductometric, amperometric and capacitance methods.

Among optical methods may particularly be mentioned those that detect mass surface concentration or refractive index, such as reflection-optical methods, including both internal and external reflection methods, e.g. ellipsometry and evanescent wave spectroscopy (EWS), the latter including surface plasmon resonance spectroscopy (SPRS), Brewster angle refractometry, critical angle refractometry, frustrated total reflection (FTR), evanescent wave ellipsometry, scattered total internal reflection (STIR), optical wave guide sensors, evanescent wave based imaging, such as critical angle resolved imaging, Brewster angle resolved imaging, SPR angle resolved imaging, etc., as well as methods based on evanescent fluorescence (TIRF) and phosphorescence.

Instruments based on SPR and FTR technology, respectively, are commercially available, i.e. BIAcore® (Jönsson et al., (1991), supra), from Pharmacia Biosensor AB, Sweden, and IAsys™ (Hodgson et al., Bio/Technology, vol. 12, jan. (1994)) from Fisons Plc., England.

A BIAcore® instrument, based on surface plasmon resonance (SPR) detection, was used in the Examples described further below. The phenomenon of SPR is well known. In brief, SPR is observed as a dip in intensity of light reflected at a specific angle from the interface between an optically transparent material, e.g. glass, and a thin metal film, usually silver or gold, and depends on among other factors the refractive index of the medium (e.g. a sample solution) close to the metal surface. A change of refractive index at the metal surface, such as by the adsorption or binding of material thereto, will cause a corresponding shift in the angle at which SPR occurs. To couple the light to the interface such that SPR arises, two alternative arrangements are used, either a metallized diffraction grating (Wood's effect), or a metallized glass prism or a prism in optical contact with a metallized glass substrate (Kretschmann effect). For further details on SPR, reference is made to WO 90/05295.

In one embodiment of the present invention, of a fraction of, or the complete, sample (e.g. amplification product) is first immobilized, followed by monitoring of wildtype or competitor sequence-specific shortening, employing a suitable enzyme such as a restriction endonuclease or ribozyme. The desired quantification of the ratios between the different nucleic acid species is obtained easily and reproducibly, since the activities of the enzymes can be monitored and controlled.

In another embodiment of the present invention, the quantification of nucleic acid fragments (e.g. PCR products) is performed by a first hybridisation. This is of special interest for the determination of the relative ratio of PCR products from competitive PCR based diagnostic applications (Lundeberg et al, Biotechniques, 10: 68-75 (1991)). In this embodiment, respective oligonucleotides specific for the different nucleic acid species are allowed to hybridize to an immobilized representative sample from the competitive PCR. The relative ratio between the nucleic acid species can then be determined from the hybridization signals which optionally may be amplified by the binding of an amplification molecule (e.g. monoclonal or polyclonal antibodies) or other substance. The signal from the hybridisation can hence be converted into quantitative information of what amounts of specific nucleic acid species are present as immobilized onto the solid support.

In still another embodiment of the present invention, the relative ratio between the nucleic acid species may be determined by quantitative monitoring of controlled primer extension using the immobilized nucleic acid fragments as templates, a suitable polymerase and free nucleotides.

Yet another embodiment of the present invention is based on the monitoring of sequence specific capture of DNA or RNA fragments via immobilized wildtype or competitor specific probes, followed by enzymatic extension or specific binding of another molecular species, such as antibody.

The invention will in the following be further illustrated by non-limiting examples with reference to the appended drawings.

SHORT DESCRIPTION OF THE DRAWINGS

Figure 1 shows the sequences, lengths and relations of synthetic oligonucleotides used in some of the Examples. For simplicity, the oligonucleotides are denoted 1 through 6. The three subfragments can be assembled into a 69 bp double stranded DNA fragment via the 3 nt protrusion ends. The G in oligonucleotide 4 marked with an arrow indicates the nucleotide determined in the mini DNA-sequencing protocol, in which a 5'-biotinylated version of oligonucleotide 6 was used.

Figure 2 is a schematic drawing of different strategies for PCR product quantification employing real-time monitoring of nucleic acid hybridisations. Optionally, the signal from the hybridisation reaction may be enhanced by binding of e.g. an antibody to a label present in the oligonucleotide used for hybridisation.

Figure 3 is a schematic view of the consecutive steps in the gene assembly mentioned in the description of Fig. 1. Panel F shows a hybridisation of oligonucleotides enabling enzymatic extension. 5'-phosphate groups of the oligonucleotides are indicated (open ring).

Figure 4 is a sensorgram from biosensor analysis of the gene assembly in Fig. 3. The capital letters correspond to the different events as outlined in Fig. 3. The numbers in the sensorgram correspond to the net response obtained in each step.

Figure 5 is a sensorgram from a DNA synthesis experiment. The DNA polymerase activities of T7 DNA polymerase and *E. coli* DNA polymerase I (Klenow fragment) were investigated in real-time in a primer extension experiment. A) Schematic overview of the steps in the experiment; B) Sensorgram obtained from the experiment using T7 DNA polymerase; C) Sensorgram obtained from the experiment using DNA polymerase I (Klenow fragment).

Figure 6 shows a cleavage experiment with the endonuclease *Xho* I. A) A schematic view of the cleavage of the immobilized double stranded DNA fragment; B) Sensorgram registered during the injection of the endonuclease *Xho* I; C) Enlargement of the upper section of the sensorgram in Figure 6B.

Figure 7 is a schematic drawing of the principles for enzymic quantification of PCR products obtained e.g from a competitive PCR strategy. A) Quantification through the monitored action by a suitable restriction endonuclease, recognizing a sequence specific for a certain nucleic acid fragment. B) Quantification through polymerase assisted extension of a hybridised oligonucleotides, specific for certain nucleic acid fragments.

Figure 8 is a schematic drawing of the DNA strands which in Example 1 were present in different ratios as immobilized on the biosensor surface. Shown also are the nucleotide sequences of the two different oligonucleotides "comp" and "wt" (each is 17 nucleotides long) used for the hybridisation experiment.

Figure 9 is a histogram showing the relative responses obtained in the hybridisation experiment in Example 1, for the different flow cells of the biosensor.

Figure 10 is a sensorgram obtained in an experiment wherein the signal from a hybridisation (left) was amplified by the binding of an antibody (right), recognizing label groups present in the 5´-ends of the oligonucleotides used for hybridisation in Example 4.

EXAMPLES

Starting materials

Oligonucleotides

Six oligonucleotides, designed for assembly into a 69 bp fragment (Fig. 1), were synthesized on an automated DNA synthesizer (Gene Assembler® Plus, Pharmacia Biotech AB, Uppsala, Sweden) according to the manufacturer's recommendations. The oligonucleotides were purified using an FPLC® pepRPC5/5 column (Pharmacia Biotech AB) (Hultman et al, Biotechniques, 1: 84-93, (1991)). Phosphorylation

was performed according to Sambrook et al (Molecular cloning: A laboratory manual, Cold Spring Harbour Laboratory Press). Oligonucleotides 1 and 6 (Example 1) were synthesized both with and without a 5′ biotin group using biotin-phosphoramidite (Clontech), for use in different examples. Other oligonucleotides used in the examples are listed below:

NIPE-8: ***Biotin***-AACACAACGCTCTACAGCAGAATTGTGAGCGGATAACAATT-3
NIPE-18: ***FITC***-CTCCTGCAGCTTCAAGAACTGTG-3′
NIPE-17: ***Biotin***-GTGATCTCCGTTTCCAATCCTGG-3′
Wt: GGGAGAAAGAGTGTCTT-3′
Comp: AATTGTTATCCGCTCAC-3

PCR

Standard PCR reactions (Hultman et al., Nucleic Acid Research (1989) 17: 4937-4946) were performed using oligonucleotides NIPE-17 (Biotin′-GTGATCTCCGTTTCCAATCCTGG-3′) and NIPE-18 (FITC-CTCCTGCAGCTTCAAGAACTGTG-3′) on the plasmid templates pRIT28-C.T. or pRIT28-C.T./comp (available at Royal Institute of Technology, Stockholm, Sweden), yielding PCR products of 97 bp.

Biosensor instrument

A BIAcore® instrument (Pharmacia Biosensor AB, Uppsala, Sweden) was used in all Examples. The responses are given in resonance units (RU). Sensor chips SA5 (research grade), precoated with approximately 4000 RU [1000 RU corresponds to approximately 1 ng/mm^2 (Jönsson et al., Biotechniques, 5: 620-627, (1991))] of streptavidin, were from Pharmacia Biosensor AB. Before use in experiments, the sensor chips were treated with several (5-6) pulses of 1 mM hydrochloric acid to pre-condition the surface (corresponding to a decrease in the signal of approximately 30-500 RU). The temperature setting of the instrument was 25 °C. The flow rate was 2 µl/min, except for the cleavage experiment (Example 4) where 1 µl/min was used.

Buffers

Running buffer : HBS (10 mM Hepes, pH 7.4, 0.15 M NaCl, 3.4 mM EDTA and 0.05% Tween® 20) or HBS*High salt* (10 mM Hepes (Sigma), pH 7.4, 0.5 M NaCl, 3.4 mM EDTA and 0.05% Tween® 20). Capturing and hybridization buffer: HBS with 0.3 M NaCl. Ligation buffer: One Phor-All-Buffer Plus, (Pharmacia Biotech AB) (10 mM Tris-acetate, pH 7.5, 10 mM magnesium acetate, 50 mM potassium acetate) supplemented with ATP and T4 DNA ligase (Pharmacia Biotech AB) to final concentrations of 1 mM and 0.1 Weiss U/µl, respectively. Extension buffer: 0.05 U /µl polymerase in 28 mM Tris-HCl, pH 7.2, 30 mM citric acid, 10 mM $MgCl_2$, 32 mM DTT, 4 mM $MnCl_2$, supplemented with all four dNTPs to final concentrations of 0.2 mM. Cleavage buffer: 0.6 U/µl of the endonuclease *Xho* I, in 10 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 10 mM $MgCl_2$ and 0.1 µg/µl BSA (bovine serum albumin).

Antibody.

The antibody used in the hybridisation signal amplification experiment was mouse anti-fluorescein isothiocyanate (FITC), DAK-FITC4 (DAKO, Denmark).

EXAMPLE 1.

Quantification of PCR products using real-time monitoring of hybridisations

The use of PCR for the detection of e.g. pathogens in clinical samples has proven to be effective. However, the high sensitivity of PCR makes it difficult to use standard protocols for a simultaneous quantification of the number of initial templates in the sample. Therefore, strategies based on the addition of titered amounts of competitive DNA for PCR co-amplification have been developed, (Lundeberg et al. (1991), BioTechniques 10: 68-75). This allows for a quantification of the initial number of sample templates, if the ratio between the different PCR products obtained in the co-amplification can be determined. This example describes the use of real-time BIA for the quantification of *Chlamydiae trachomatis*-

specific PCR-products, using a hybridization strategy. The general quantification strategy is outlined in Fig. 2.

The PCR-products to be quantified were obtained from PCR amplification of plasmid DNA harboring a wild type *Chlamydiae trachomatis* sequence or a corresponding sequence with an internal, genetically engineered, *lac*-operator sequence (competitor) (Fig. 8). Thus, the two resulting 97 bp long PCR products differ in an internal stretch of 21 nt, used as the specific hybridisation target sequences. These PCR products were pre-mixed in four different ratios and subsequently immobilized onto a streptavidin Sensor Chip surface (SA5), utilizing a biotin group present in one of the PCR primers.

The immobilizations were performed by injections of 45 µl of the four different PCR product mixtures (200 nM DNA) in 0.5 M NaCl over the four surfaces at a flowrate of 1 µl/min. This resulted in the immobilization of approx. 2200 RU DNA in each flow cell (Fc) with calculated ratios between wild type DNA and competitor DNA of:

Flow cell	*wt/competitor*
Fc1:	70/30
Fc2:	30/70
Fc3:	100/0
Fc4:	0/100

In order to be able to experimentally verify these ratios by a hybridisation-strategy employing wildtype- and competitor-specific oligonucleotides, the double stranded immobilized DNA was made single stranded using 1 mM HCl (10 µl at a flow rate of 5 µl/min). The efficiency of these strand separations were approximately 107%. The high value can be explained by a simultaneous small loss of loosely bound double stranded DNA. Employing this relatively mild acid eluent, the streptavidin surface is almost unaffected, which is in contradiction to strand separation using NaOH (e.g. 50 mM). The resulting amounts of single stranded DNA for the different mixtures, and hence template for hybridization, were approximately 1000 RU.

The subsequent hybridizations were performed at 25°C with an oligonucleotide concentration of 10 µM in HBS with 0.5 M NaCl (30 µl at a flowrate of 5 µl/min), using the wild type-specific oligonucleotide (wt) and the competitor-specific oligonucleotide (comp), respectively, which were both 17 nucleotides long (Fig. 8). Each hybridisation was repeated seven times to allow a statistical evaluation of the results. Regenerations of the single stranded templates were performed between each run using 1 mM HCl. The hybridisations were performed at different oligonucleotide concentrations to assure a saturation of the single stranded templates.

The results from the hybridisation experiments at the four different surfaces are shown in Fig. 9 and Table 1 below and demonstrate that the values are highly reproducible with standard deviations below 1 RU. In addition, the obtained experimental data for the determination of the ratios between wildtype and competitor PCR products at the different surfaces show a close correlation with the expected values from the premixing. This suggests that this technique can be used with high reliability for the quantification of specific PCR products in a background of other fragments. Therefore, for the determination of the initial number of PCR templates (e.g. bacterial or viral genomes), the hybridisation strategy using surface plasmon resonance (SPR) detection shown here can be an alternative to methods based on electrophoresis or enzymatic detection principles.

In order to enhance the signals obtained in the hybridisation, different methods can be used.

Signal amplification using monoclonal antibodies specific for FITC-labeled oligonucleotides will be described in Example 2 below.

A subsequent extension of the annealed oligonucleotides employing a DNA polymerase (e.g. Klenow DNA polymerase I) together with free nucleotides also results in a significant signal amplification. Such DNA

synthesis, which is included in the general quantification strategy outlined in Fig. 7, will be described in Example 3 below.

Enzymatic sequence-specific shortening, also included in the quantification strategy shown in Fig. 7, will be described in Example 4 below.

EXAMPLE 2

Hybridisation-signal amplification.

A template for hybridisation to the oligonucleotide NIPE-18 was obtained by a first immobilization (at a flowrate of 1 µl/min) onto a streptavidin coated sensor chip surface (SA-5) of approximately 2000 RU of a 97 PCR product (45 µl of 200 nM in 0.5 M NaCl) obtained from PCR on plasmid pRIT28-C.T., using oligonucleotides NIPE-17 and NIPE-18. After injection of 20 µl of 1 mM HCl, selectively eluting the un-biotinylated strand, approximately 1100 RU of a single stranded template for the FITC-labeled oligonucleotide NIPE-18 was obtained. A solution of NIPE-18 (2 µM) was subsequently injected, resulting in the hybridisation of 196 RU to the single stranded template. In order to investigate if this signal could be amplified by the use of the FITC label present at the 5´-end of oligonucleotide NIPE-18, a 300 nM solution of a mouse anti-FITC monoclonal antibody was injected (30 µl). As shown in the sensorgram of Fig. 10, this resulted in an increase in the signal by approximately 1200 RU. Thus, the signal from a hybridisation can be amplified by at least a factor ten, by the use of a reagent capable of selectively recognizing a label present in the hybridising nucleic acid fragment.

EXAMPLE 3

DNA synthesis

The oligonucleotides outlined in Fig. 1 can be assembled in a stepwise manner into a 69 bp double stranded DNA fragment from three smaller fragments with overlapping protrusions. The experiments, outlined in Fig. 3 (A-E), were monitored in real-time using biosensor technology adapted for streptavidin-biotin chemistry. The corresponding sensorgram is shown in Fig. 4. A prewashed

sensor chip SA5 containing approximately 3500 RU of covalently coupled streptavidin was used to capture 5′-biotinylated oligonucleotide 1 (Fig. 3 (A)), injected (30 µl, 2 pmole/µl) over the surface. As appears from the result shown in Fig. 4 (A), the capture of this oligonucleotide (Fig. 3 (A)) is rapid and a steady level is quickly reached. The amount bound (1400 RU), corresponds to a stoichiometry of 2.9 bound oligonucleotides (M_W = 8.3 kDa) per tetramer of streptavidin (M_W = 60 kDa, for the tetramer), which theoretically binds 4 biotins. This shows that a good accessibility to the binding sites of streptavidin is achieved in the immobilization. Injections with a two-fold higher concentration of the oligonucleotide did not increase the uptake (data not shown), indicating a saturation of the streptavidin surface. The captured single stranded biotinylated 25-mers were subsequently used for the annealing of the complementary strand (Fig. 3(B)). Oligonucleotide 2 was injected (30 µl, 2 pmole/µl) and the uptake was registered. As can be seen in the first part of the injection in the resulting sensorgram (Fig. 4 (B)), the binding of this interaction is slower than for the streptavidin-biotin interaction. The increase in response (760 RU) corresponds to an apparent efficiency in this step of 64%. Injections with higher concentrations of the oligonucleotide did not result in higher signals (data not shown). No detectable binding was seen from injections with a non-complementary oligonucleotide (data not shown), indicating that the observed annealing was specific.

In order to continue the assembly of the DNA fragment, oligonucleotides 4 (non-phosphorylated) and 3, were prehybridized and subsequently injected (40 µl, 2 pmole/µl) over the surface. This fragment contains a 3 nt protrusion which is complementary to the sequence present at the free end of the immobilized fragment, consisting of oligonucleotides 1 and 2. No increase in the signal was observed when the injection was performed without DNA ligase (data not shown). In contrast, when injecting the fragment, with the addition of T4 DNA ligase, a significant

binding was recorded by the real-time analysis, as demonstrated in Fig. 4 (C). This shows that the binding via the overlapping region of 3 bp is not sufficient for the formation of stable non-covalent complexes at this temperature (25° C), but the DNA ligase allows a transient interaction followed by covalent attachment. The shape of the curve observed during the injection of the DNA/ligase mixture indicates a bi-phasic reaction (Fig. 4 (C)). The gradual increase of the signal, at the end of the injection pulse, also suggests that the equilibrium is not reached during the injection period (20 minutes). A subsequent injection (8 µl) of SDS (0.05%) washing solution resulted in a 200 RU drop in response, as can be seen in Fig. 4 (C-D), demonstrating the necessity to eliminate background response from T4 DNA ligase attached to the DNA for correct evaluation of the result. A single injection of the SDS solution was sufficient, since repeated injections did not further affect the signal (data not shown). From the analysis, a net response of 930 RU was calculated, corresponding to a ligation efficiency of 64 % (930/1450). In a control experiment, a fragment containing a non-complementary protrusion was injected together with T4 DNA ligase; no change in the signal was observed (data not shown).

A second ligation step, completing the assembly, was performed using prehybridized oligonucleotides 5 and 6 (Fig. 3 (D))(40 µl, 2 pmole/µl). Note that oligonucleotide 4 lacks a 5'-phosphate group and ligation can therefore only occur on one of the strands. A plateau value was reached after approximately 13 minutes, (Fig. 4 (D)), suggesting that this ligation step reaches equilibrium more rapidly than the previous ligation (Fig. 4 (C)). Interestingly, the shape of the curve indicates that this ligation is not a bi-phasic event. This might be explained by the absence of a 5´-phosphate group in oligonucleotide 4, resulting in the formation of a single phosphodiester bond per fragment, rather than two in the preceding ligation. The efficiency of this second ligation step,

calculated from the response after the SDS washing (680 RU) was 63%. Thus, the overall efficiency of this still non-optimized assembly of the 69 bp DNA fragment, calculated from the 1400 RU of initially immobilized biotinylated oligonucleotide, was 26% (0.64 x 0.64 x 0.63). The assembly was followed by strand specific elution of the non-biotinylated strand, (Fig. 3 (E)). A short pulse of alkali (8 µl, 50 mM NaOH) resulted in a significant decrease in the signal (Fig. 4 (E)). The amount released (1780 RU) suggests efficient release of the non-immobilized strand. The amount of full length single stranded DNA present on the surface after this alkali pulse is approximately 970 RU. Note that the eluted DNA consists of two separate fragments, due to the absence of a phosphate group in oligonucleotide 4.

A clear difference in the curve shapes was observed for the two ligation steps in the gene assembly experiment. These ligation steps take place at different relative distances from the surface and involve the formation of two and one phosphodiester bond(s), respectively. However, when analysing results from similar experiments (data not shown), a general two-phasic curve for ligations involving the formation of two phosphodiester bonds can be seen, regardless of the position for ligation. The successful real-time monitoring of the assembly of the 69 bp double stranded model DNA fragment shows the possibilities of using biosensor technology for the optimisation of critical steps during the procedure, which is not possible for other solid-phase gene assembly methods (Hostomsky et al., Nucleic Acids Res. 12: 4849-4856, (1987), Ståhl et al., Biotechniques 3: 424-434 (1993)). The short protrusion ends (3 nt) of the fragments were sufficient for the formation of substrates for the ligase, even at 25°C, a temperature well above the calculated melting temperatures (Tm) for the annealing stretches (7°C and 8°C, respectively).

The results from the gene assembly experiment show that an enzymatic action upon DNA can be monitored with

real-time BIA, using standard flow-rates and injection volumes. To investigate if also the performance of other nucleic acid modifying enzymes could be monitored using BIA, the polymerase activities of T7 DNA polymerase (Pharmacia Biotech AB, Uppsala, Sweden) and DNA polymerase I (Klenow fragment) (Boehringer Mannheim, Germany) were investigated. In order to prepare suitable substrates for both of these primer dependent enzymes, oligonucleotide 6 (Fig. 1) (20 µl, 2 pmole/µl) was hybridized to the 3'-end of the immobilized 69 nt single stranded DNA (810 RU), (Fig. 5 (A-I)). After injection (10 µl) of T7 DNA polymerase in extension buffer containing dNTPs, (Fig. 5 (A-II)), a gradual increase of the signal was seen during the entire sample pulse. In the following flow of running buffer, a slow dissociation from the surface was observed, (Fig. 5 (B-II)). A subsequent pulse (8 µl) of SDS solution (0.05%) resulted in a further decrease to a stable value, (Fig. 5 (B)), which was not changed by further injections. In a control experiment, in which T7 DNA polymerase was injected over a plain streptavidin surface, no interaction was observed (data not shown), indicating that the previous interaction was DNA dependent. When T7 DNA polymerase was injected over the single stranded DNA/primer substrate, without nucleotides present in the buffer, a gradual accumulation was seen, but with no increase of the signal after SDS washing (data not shown). Therefore, the gradual increase of the signal seen during the entire pulse of T7 DNA polymerase in the extension experiment can be interpreted as a sum of signals from extension and accumulation of T7 DNA polymerase on the DNA. The resulting SPR response corresponds to a near complete extension of all the hybridized primers. A final injection (8 µl) of alkali (50 mM NaCl), results in a decrease of approximately 680 RU, which correlates well with a complete release of the "second strand" DNA made up from extended primers (Fig. 5 (AB-III)).

In a subsequent study, DNA polymerase I (Klenow fragment) was analysed using the same experimental setup.

For this polymerase, a gradual increase of the RU signal was seen only during the first minute of injection, after which the signal rapidly declines to a steady plateau value, (Fig. 5 (C-II)). In the following flow of running buffer, no significant decline was seen and the subsequent SDS pulse only resulted in a minor decrease of the signal, (Fig. 5 (C)). Also in this case, the resulting signal decrease after the final alkali injection corresponds to a complete release of the "second strand" DNA, obtained from an extension of the hybridized primers (approximately 680 RU). The complete dissociation from the immobilized templates seen for the Klenow polymerase already after approximately one minute of injection indicates that the DNA synthesis is completed after that period of time.

EXAMPLE 4

Cleavage with endonuclease

Oligonucleotide 1 contains the contribution from one strand to the recognition sequence for the endonuclease *Xho* I (Fig. 1). Thus, double stranded DNA obtained by extension towards the sensor chip surface using the priming outer oligonucleotide 6 together with Klenow polymerase, contains the complete recognition site (Fig. 6 (A)). In order to evaluate if also an endonuclease activity could be monitored using BIA, a 45 minutes pulse of *Xho* I present in its recommended assay buffer was injected over 1310 RU of such 69 bp substrate DNA (Fig. 6 (B)), obtained from a gene assembly experiment (data not shown). This resulted in an initial increase of the signal, followed by a slow but significant decline. After the injection of endonuclease, the response level decreased with approximately 900 RU. A subsequent SDS pulse (8 µl, 0.05%) did not result in any further decrease. Taking into account that a fragment of 16/20 nucleotides is left on the surface after cleavage, the efficiency at this temperature (25°C) was calculated to 93 %. An enlargement of the sensorgram corresponding to the cleavage phase clearly demonstrates a sigmoidal curve shape for this enzyme-substrate reaction (Fig. 6 (C)).

The data from the *Xho* I endonuclease cleavage experiment show that the action of endonucleases can be monitored and that the signals can be interpreted in a quantitative manner.

TABLE 1

Immobilized DNA:	wild type (70%) + competitor (30%)	wild type (30%) + competitor (70%)	wild type (100%)	competitor (100%)
comp [RU]: wt [RU]:	33.1 ± 1.0 88.3 ± 0.5	77.8 ± 0.3 41.0 ± 0.2	3.9 ± 0.5 124.8 ± 0.3	104.8 ± 0.3 7.3 ± 0.4
comp + wt :	121.4	118.8	128.7	112.0
% wt : % comp :	73 27	35 65	97 3	7 93

The meanvalues and standard deviations are calculated from eight separate hybridizations.

CLAIMS

1. A process for the quantification of a target nucleic acid in a sample, comprising the steps of:

(i) adding to the sample containing said target nucleic acid a known amount of a competitor nucleic acid;

(ii) amplifying both the competitor and target nucleic acids using reagents capable of amplifying the competitor and target nucleic acids in parallel;

(iii) immobilizing the amplified nucleic acids onto a biosensor sensing surface; and

(iv) subjecting the respective immobilized nucleic acids to a biospecific interaction or interactions, and from the changes in a property of the sensing surface caused by the interactions of the respective nucleic acids determining the relative amounts of the target and competitor nucleic acids to thereby determine the amount of said target nucleic acid in said sample.

2. The process according to claim 1, wherein said biospecific interactions comprise hybridisation of competitor- and target-specific oligonucleotides.

3. The process according to claim 1, wherein said biospecific interactions comprise specific restriction endonuclease cleavage of the immobilized nucleic acid species.

4. The process according to claim 1, wherein said biospecific interactions comprise nucleic acid synthesis on the immobilized nucleic acid species as templates, employing as starting primers oligonucleotides capable of selective hybridisation to the target and competitive nucleic acids, respectively.

5. The process according to claim 2, wherein the sensing surface response is amplified by antibody capable of

selective recognition of the oligonucleotides used in the hybridisation.

6. The process according to any one of the preceding claims, wherein said nucleic acid is DNA or RNA.

7. The process according to any one of the preceding claims, wherein said reagents used in the amplification comprise oligonucleotide primers capable of hybridising to sequences shared by the two nucleic acid species.

8. The process according to any one of the preceding claims, where the amplification comprises using the polymerase chain reaction (PCR).

9. The process according to any one of claims 1 to 6, wherein said amplification comprises using Q_b replicase.

10. The process according to any one of the claims 1 to 7, wherein said amplification comprises using the ligase chain reaction (LCR).

11. The process according to any one of the preceding claims, wherein said biospecific interactions at the sensing surface are monitored in real-time.

12. The process according to any one of the preceding claims, wherein said measured property of the sensing surface is refractive index.

13. The process according to claim 12, wherein the measurement of refractive index is based on evanescent wave sensing, such as surface plasmon resonance (SPR) or frustrated total reflection (FTR).

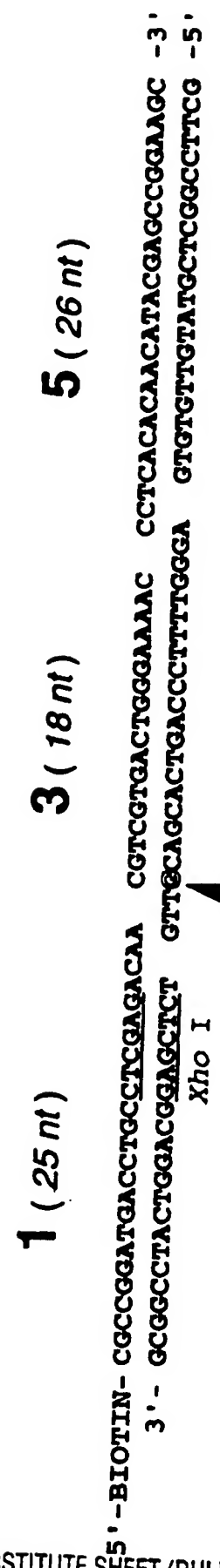

5'-BIOTIN- CGCCGGATGACCTGCCTCGAGACAA CGTCGTGACTGGGAAAAC CCTCACACAACATACGAGCCGGAAGC -3'
3'- GCGGCCTACTGGACGGAGCTCT GTTGCAGCACTGACCCTTTTGGGA GTGTGTTGTATGCTCGGCCTTCG -5'
Xho I
1 (25 nt)
2 (22 nt)
3 (18 nt)
4 (24 nt)
5 (26 nt)
6 (23 nt)

FIG. 1

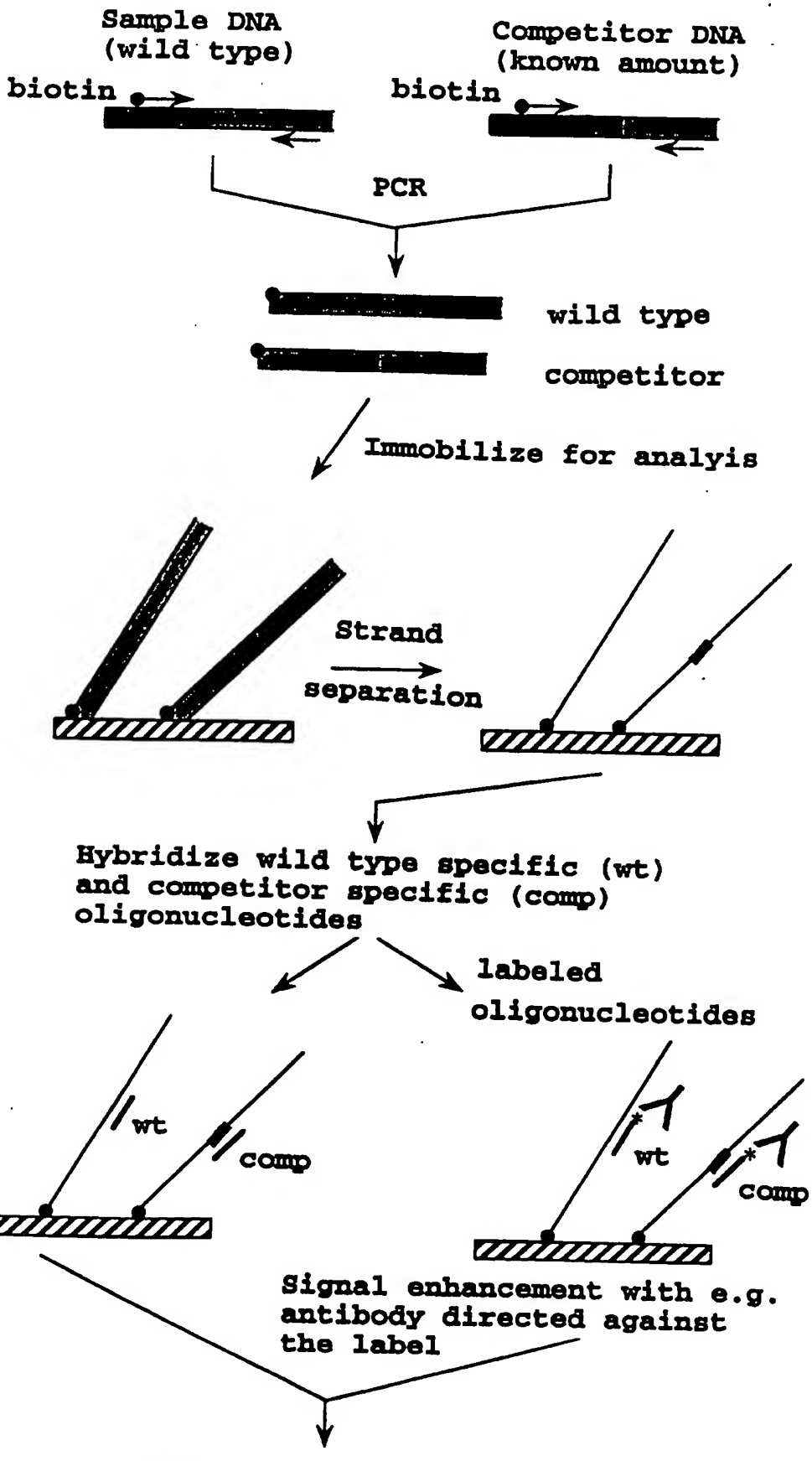

Sample DNA
(wild type)
biotin
Competitor DNA
(known amount)
biotin
PCR
wild type
competitor
Immobilize for analyis
Strand
separation
Hybridize wild type specific (wt)
and competitor specific (comp)
oligonucleotides
labeled
oligonucleotides
wt
comp
wt
comp
Signal enhancement with e.g.
antibody directed against
the label
wild type/competitor
ratio

FIG. 2

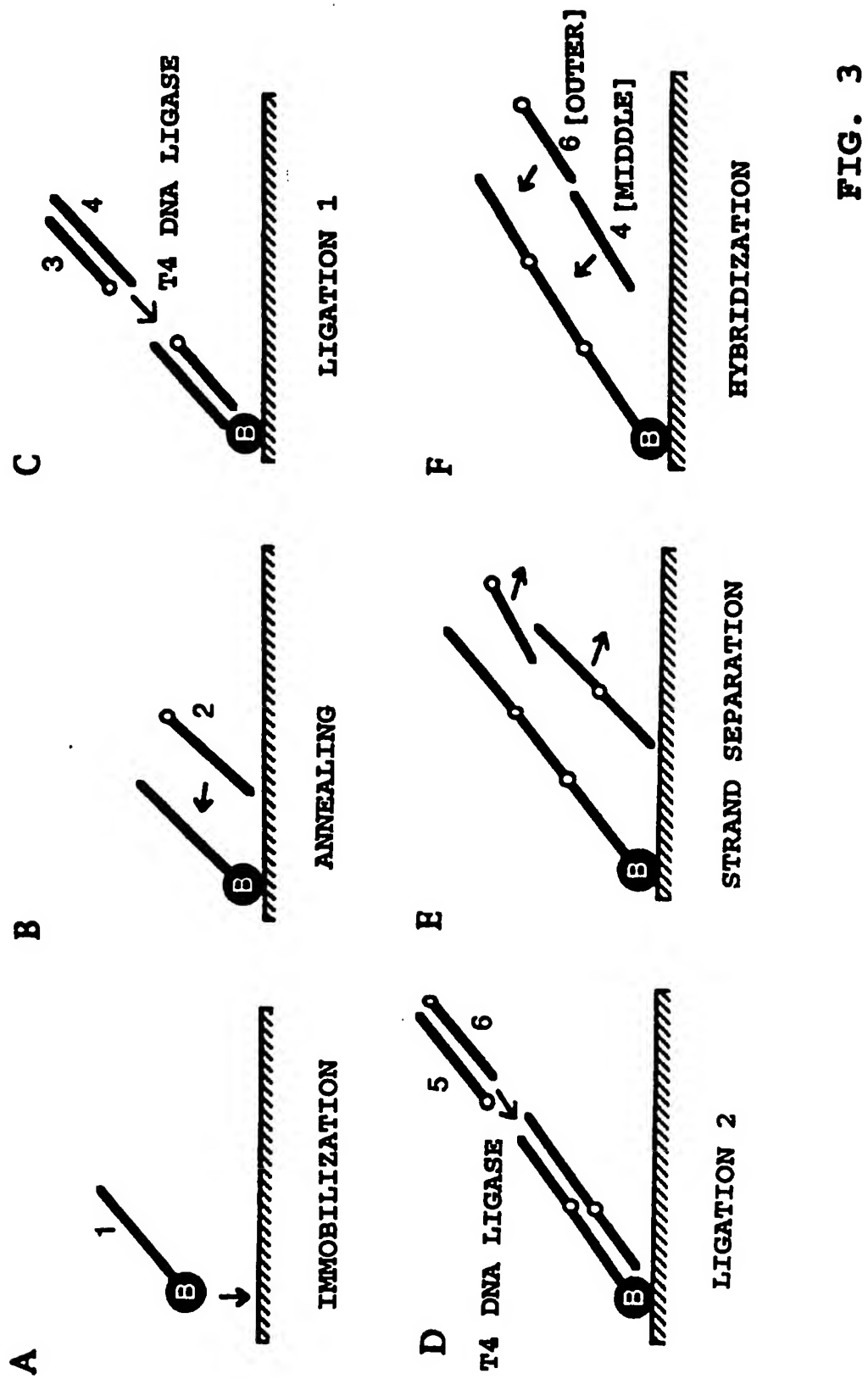

A
1
B
IMMOBILIZATION
B
2
ANNEALING
C
3
4
T4 DNA LIGASE
LIGATION 1
D
5
6
T4 DNA LIGASE
LIGATION 2
E
STRAND SEPARATION
F
6 [OUTER]
4 [MIDDLE]
HYBRIDIZATION

FIG. 3

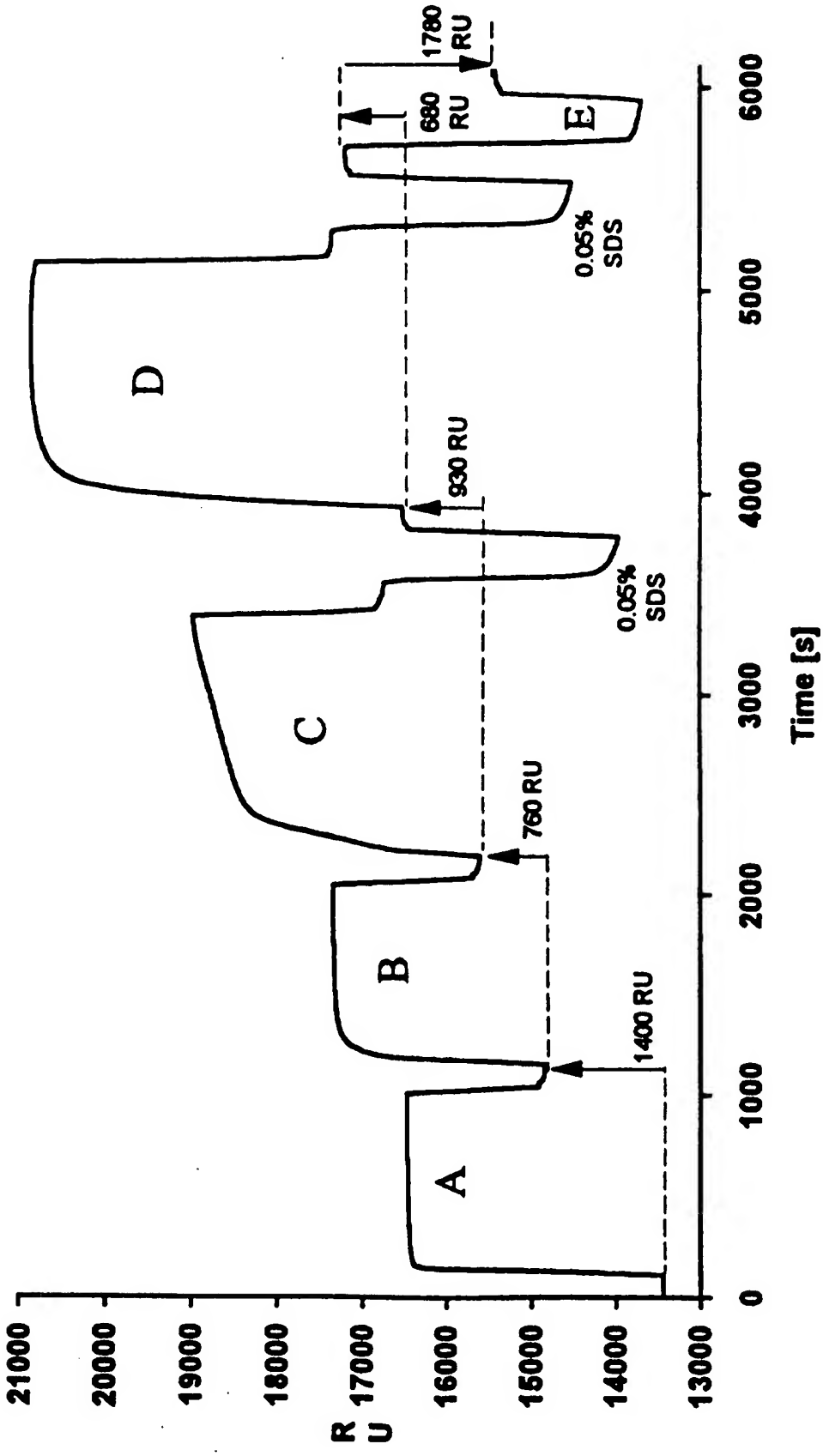
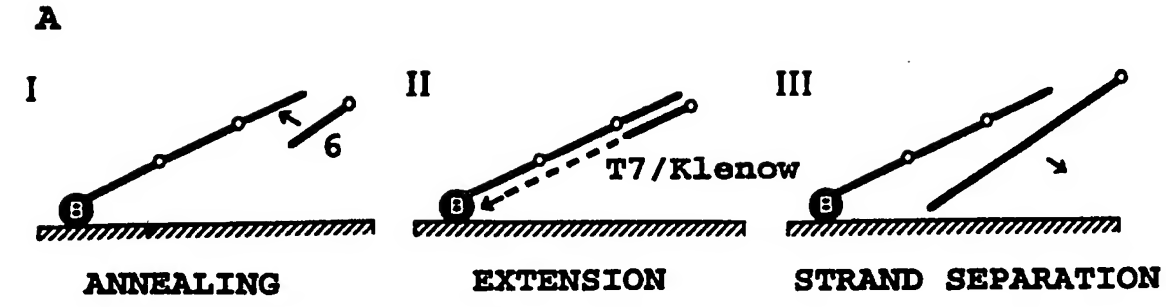
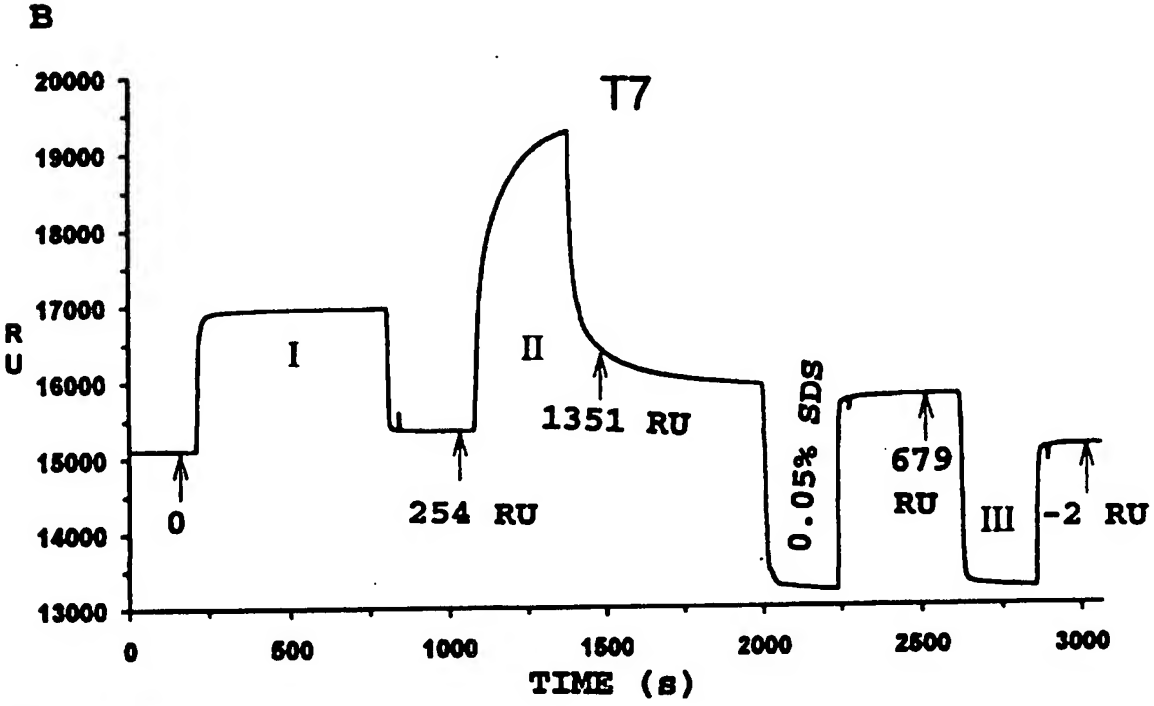

RU
21000
20000
19000
18000
17000
16000
15000
14000
13000
0
1000
2000
3000
4000
5000
6000
Time [s]
A
B
C
D
E
1400 RU
760 RU
930 RU
680 RU
1780 RU
0.05% SDS
0.05% SDS

FIG. 4


A
I
II
III
6
T7/Klenow
ANNEALING
EXTENSION
STRAND SEPARATION


B
T7
RU
TIME (s)
I
II
III
0
254 RU
1351 RU
0.05% SDS
679 RU
-2 RU

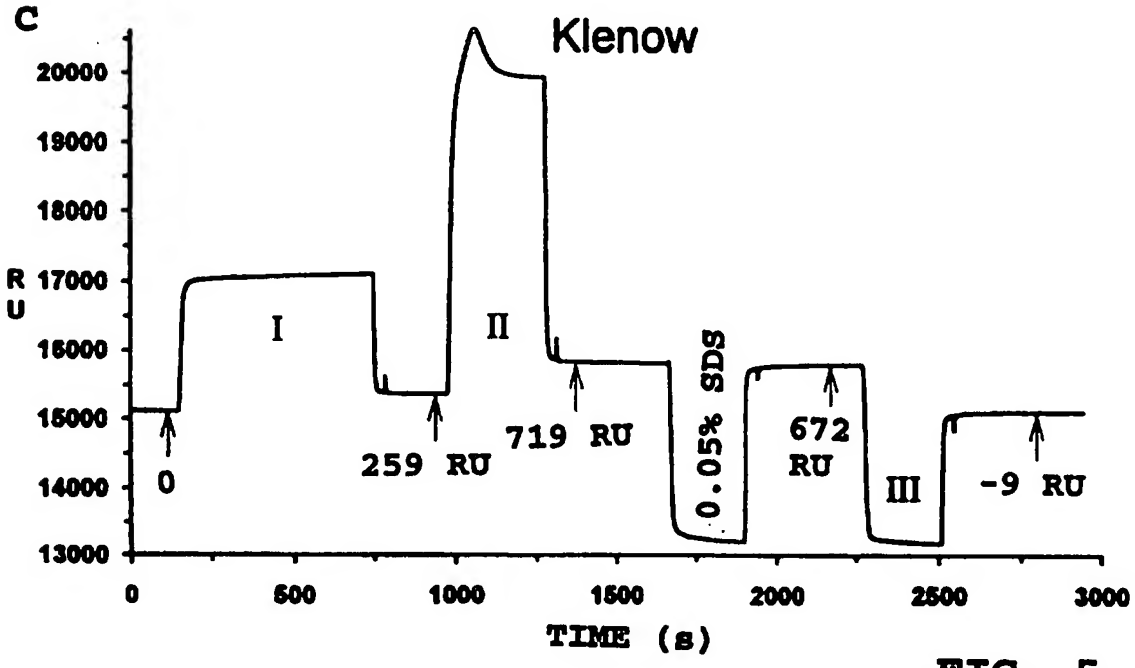

C
Klenow
RU
TIME (s)
I
II
III
0
259 RU
719 RU
0.05% SDS
672 RU
-9 RU

FIG. 5

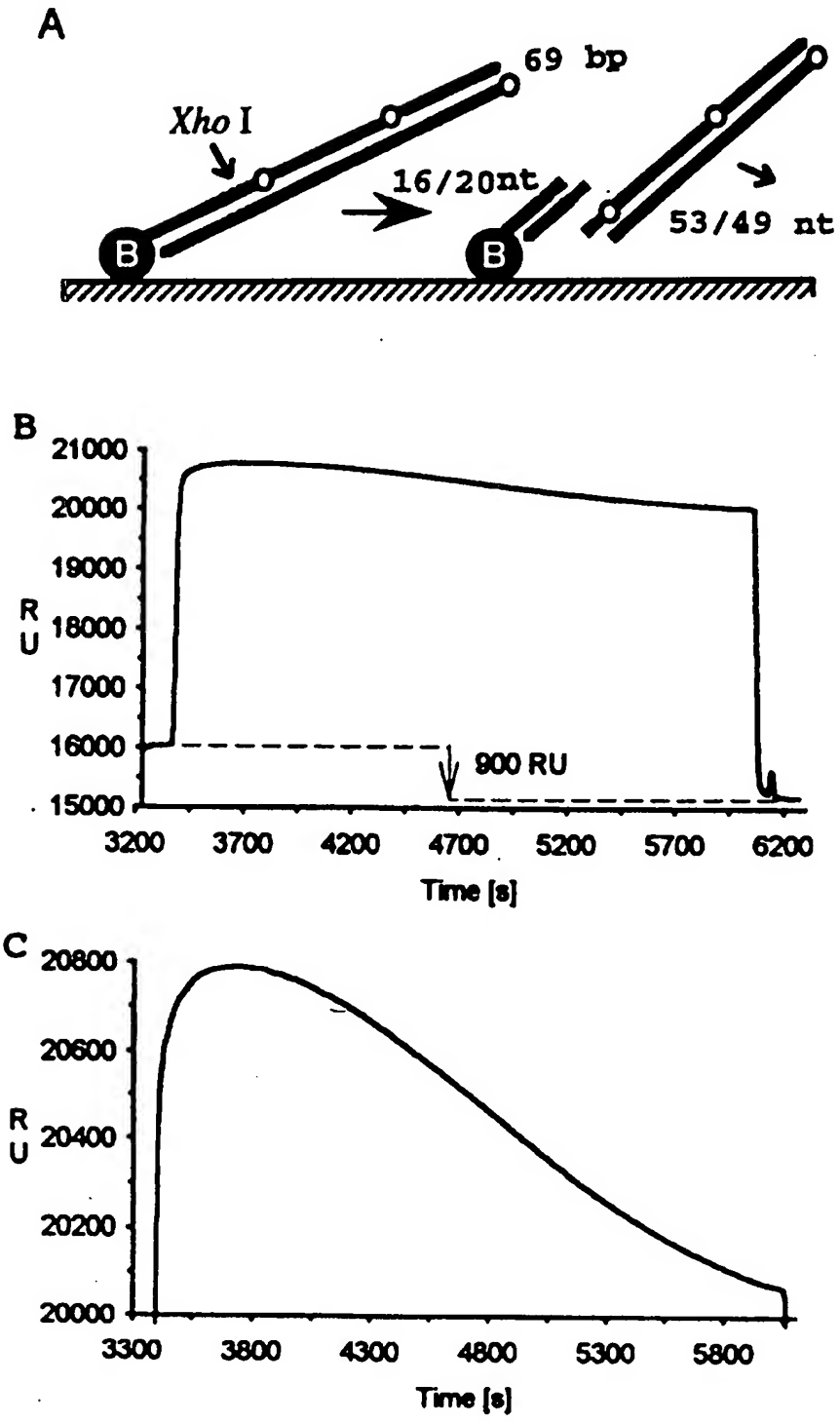

A
69 bp
Xho I
16/20nt
53/49 nt
B
B
21000
20000
19000
18000
17000
16000
15000
RU
900 RU
3200
3700
4200
4700
5200
5700
6200
Time [s]
C
20800
20600
20400
20200
20000
RU
3300
3800
4300
4800
5300
5800
Time [s]

FIG. 6

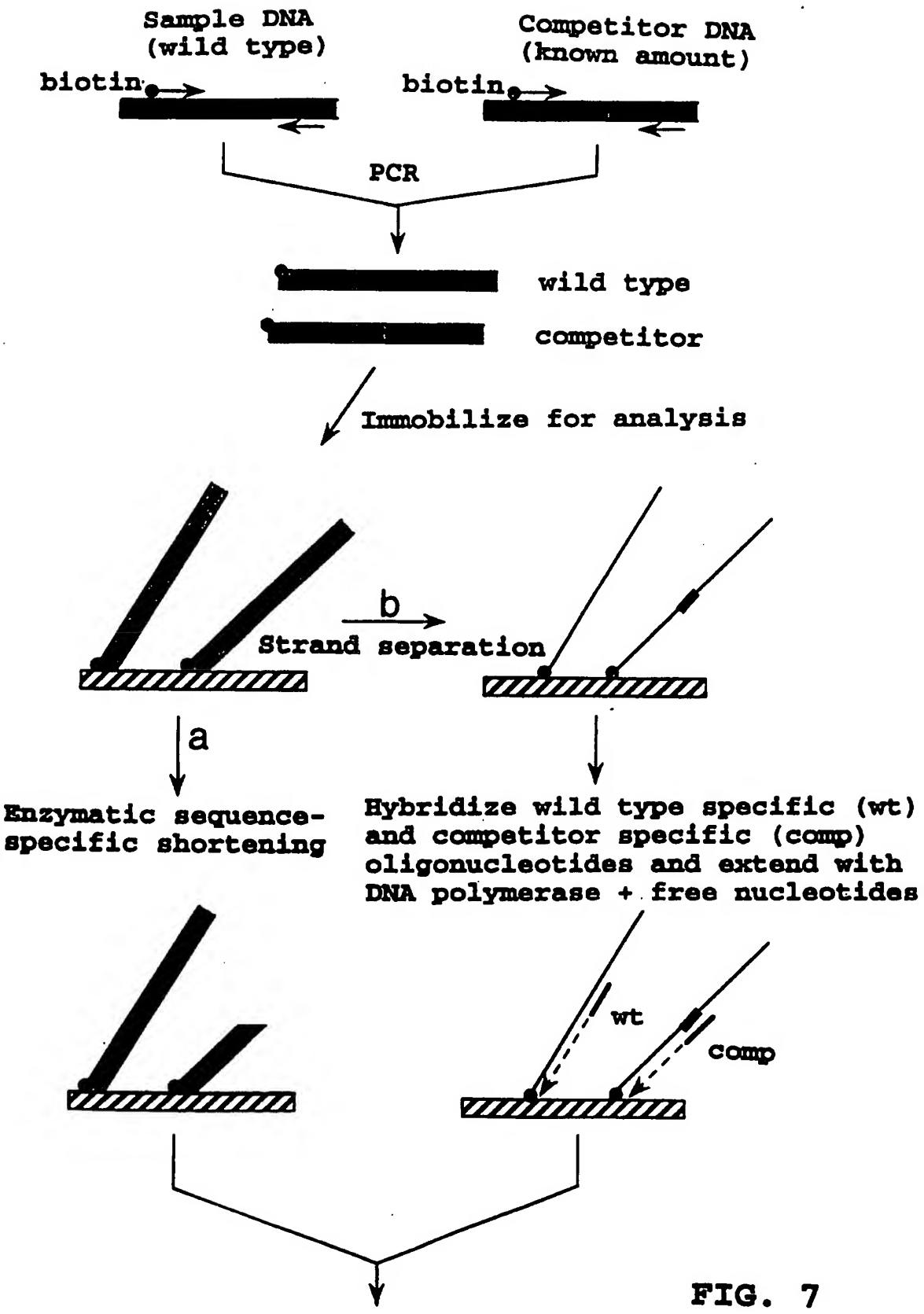

Sample DNA
(wild type)
Competitor DNA
(known amount)
biotin
biotin
PCR
wild type
competitor
Immobilize for analysis
b
Strand separation
a
Enzymatic sequence-specific shortening
Hybridize wild type specific (wt) and competitor specific (comp) oligonucleotides and extend with DNA polymerase + free nucleotides
wt
comp
wild type/competitor ratio

FIG. 7

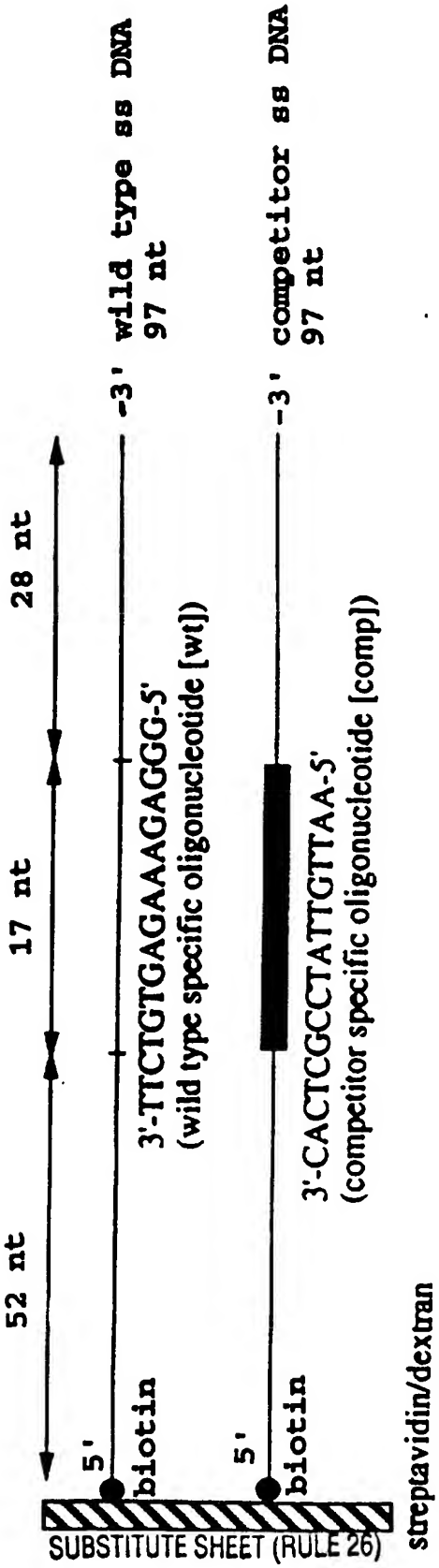

52 nt
17 nt
28 nt
5'
biotin
5'
biotin
3'-TTCTGTGAGAAAGAGGG-5'
(wild type specific oligonucleotide [wt])
3'-CACTCGCCTATTGTTAA-5'
(competitor specific oligonucleotide [comp])
streptavidin/dextran
-3' wild type ss DNA
97 nt
-3' competitor ss DNA
97 nt

FIG. 8

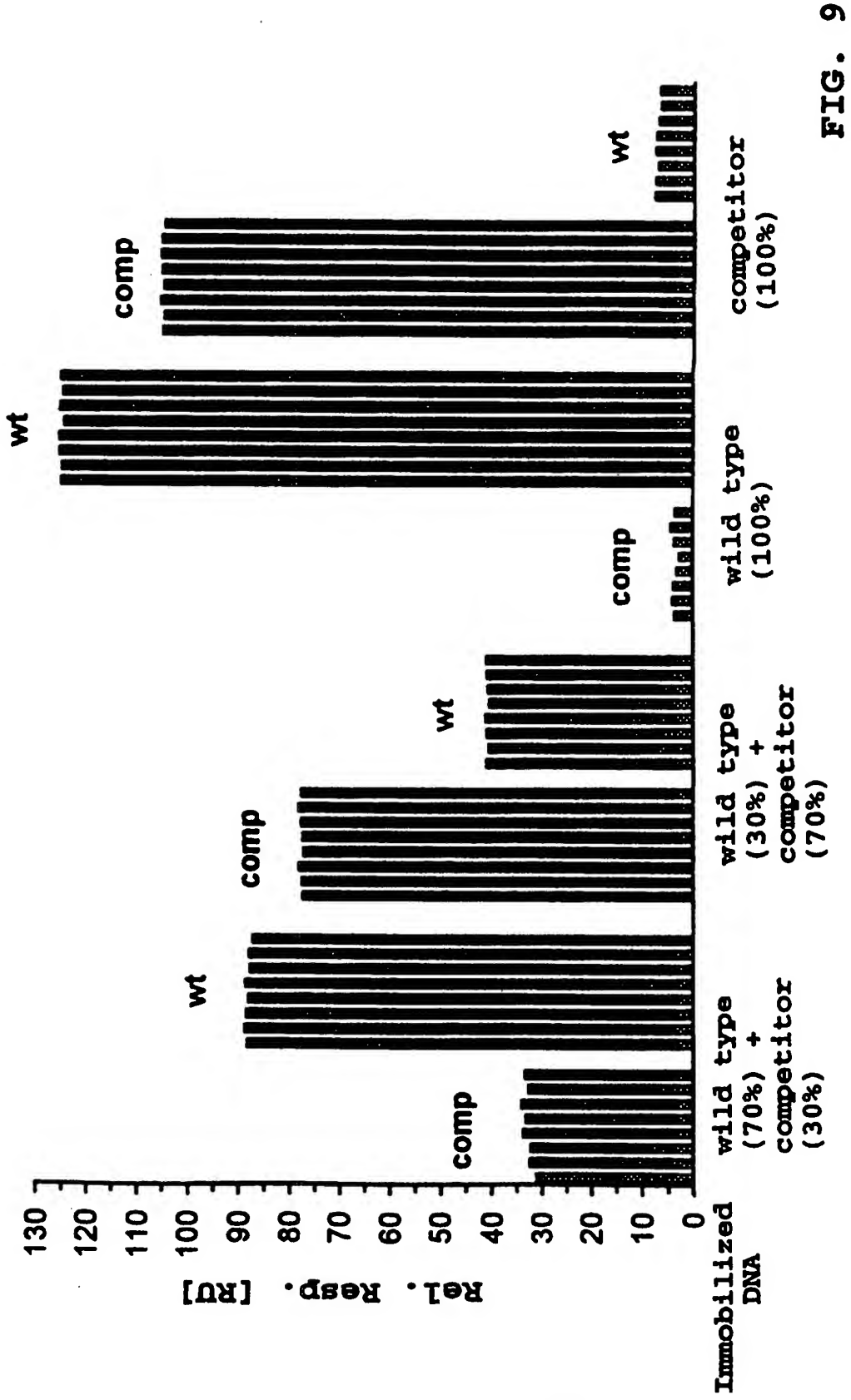

Rel. Resp. [RU]
130
120
110
100
90
80
70
60
50
40
30
20
10
0
Immobilized DNA
wild type (70%) + competitor (30%)
wild type (30%) + competitor (70%)
wild type (100%)
competitor (100%)
wt
comp

FIG. 9

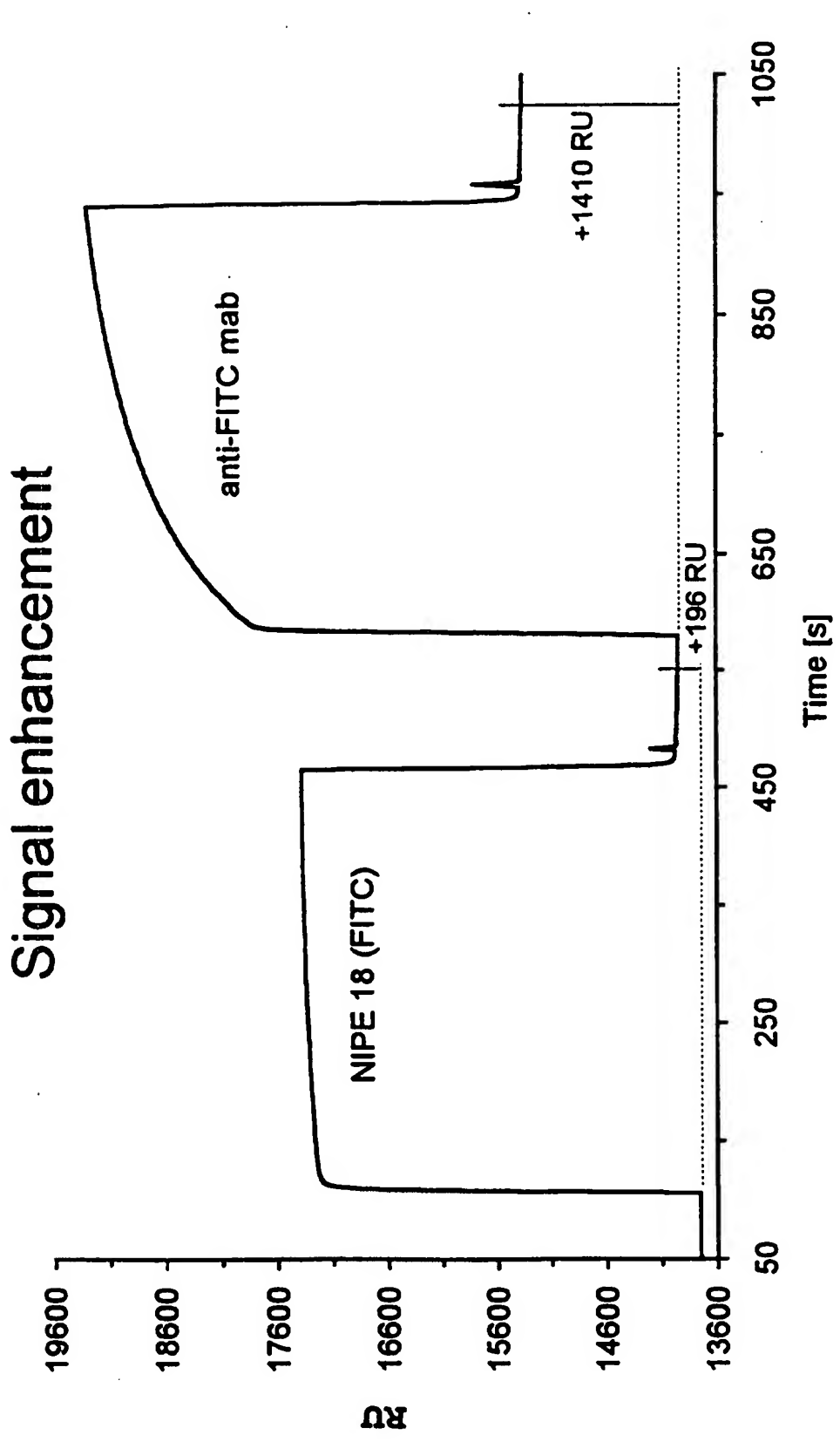

Signal enhancement
anti-FITC mab
NIPE 18 (FITC)
+1410 RU
+196 RU
RU
Time [s]
19600
18600
17600
16600
15600
14600
13600
50
250
450
650
850
1050

FIG. 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 95/01077

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12Q 1/68, G01N 33/543
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12Q, G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EDOC, WPI, PAJ, MEDLINE, BIOSIS, DERWENT BIOTECHNOLOGY ABSTRACTS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9409156 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA), 28 April 1994 (28.04.94), page 8 - page 11	1-10
Y	--	11-13
Y	Tibtech, Volume 9, October 1991, Schwarz et al, "Detection of nucleic acid hybridization using surface plasmon resonance" page 339 - page 340	11-13
	--	

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 January 1996	16 -01- 1996
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Patrick Andersson Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

11/12/95

International application No.
PCT/SE 95/01077

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 9409156	28/04/94	NONE	